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(54) Title: A METHOD FOR MASS PRODUCTION OF TAXOL BY SEMI-CONTINUOUS CULTURE													
(57) Abstract <p>The present invention relates to a method for mass production of taxol by semi-continuous culture of <i>Taxus</i> genus plant with a high yield. According to the present invention, taxol can be prepared with a high yield, by employing semi-continuous culture of <i>Taxus</i> genus plant cell, which comprises: (i) inoculating <i>Taxus</i> genus plant cell on a medium containing 1 to 10 % (w/v) sugar, and incubating it; and, (ii) transferring 1/10 to 1/2 volume of the culture obtained in the step (i) to a fresh medium and repeating the step (i), adding 1 to 10 % (w/v) sugar to the remnant culture and incubating to the time of maximum production of taxol.</p>													
<table border="1"> <caption>Data from Taxol Content Bar Chart</caption> <thead> <tr> <th>Culture Cycle</th> <th>Taxol Content (mg/l)</th> </tr> </thead> <tbody> <tr> <td>Cycle 1</td> <td>~110</td> </tr> <tr> <td>Cycle 2</td> <td>~175</td> </tr> <tr> <td>Cycle 3</td> <td>~220</td> </tr> <tr> <td>Cycle 4</td> <td>~280</td> </tr> </tbody> </table>				Culture Cycle	Taxol Content (mg/l)	Cycle 1	~110	Cycle 2	~175	Cycle 3	~220	Cycle 4	~280
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**A METHOD FOR MASS PRODUCTION OF TAXOL
BY SEMI-CONTINUOUS CULTURE**

BACKGROUND OF THE INVENTION

5

Field of the Invention

The present invention relates to a method for mass production of taxol from TAXUS genus plant cell culture, 10 more specifically, to a method for mass production of taxol by semi-continuous culture of TAXUS genus plant cell with a high yield.

Description of the Prior Art

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Taxanes are diterpene compounds containing a taxane skeleton. For example, taxol is famous as the first identified compound with a taxane ring which was isolated from the bark of the pacific yew, TAXUS brevifolia, which 20 is effective for the treatment of leukemia and cancer. Recently, it has been reported that: taxol is capable of curing approximately 30%, 50% and 20% of ovarian, breast and lung cancer patients, respectively, by inhibiting depolymerization of microtubules (see: E.K. Rowinsky et al., 25 *J. Natl. Cancer. Inst.*, 82:1247-1259(1990)).

On the other hand, total chemical synthesis, semi-synthesis and extraction methods have been employed to prepare taxol.

The total chemical synthesis method, however, has not 30 been practically applied in the art, since it requires very expensive chemical reagents and the yield is not so high, which can be expected from the complicated chemical structure of taxol.

The semi-synthesis method employing precursors such as 35 10-deacetylbbaccatin III, has revealed some drawbacks since it essentially entails complicated and multiple steps of isolating and purifying the taxol precursors from TAXUS

genus plant and transforming the precursors to taxol.

In this regard, the extraction method by which taxol can be isolated from Taxus genus plants in a direct manner, has prevailed in the art, since it has the advantage of 5 economy. However, the said method has proven to be less satisfactory in the sense that it essentially requires a large amount of yew trees to purify taxol, which finally gives rise to the serious environmental disruption.

Accordingly, the ability of total chemical synthesis, 10 semi-synthesis and extraction method to supply taxol for world-wide chemotherapeutic use is not assured; and, there are strong reasons for exploring and developing alternative means of taxol production.

As a promising alternative to solve said problems, the 15 cell culture method for taxol production has been proposed in the art.

The cell culture-based process for taxol production, unlike the prior art, has the following advantages as followings: first, taxol can be produced in a steady 20 manner, regardless of fluctuation of supply of yew plants due to the damage by blight and harmful insects, etc.; secondly, cell cultures can be propagated in large bioreactors, from which taxol can be massively produced by manipulating culture conditions; thirdly, cell cultures 25 produce a simpler spectrum of compounds compared to other prior art methods, considerably simplifying separation and purification; fourthly, a cell culture process can adapt quickly to rapid changes in demand better than the other methods; fifthly, a cell culture process can produce taxol 30 as well as taxane precursors such as baccatin that can be converted to taxol.

Methods for producing taxol by utilizing cultured plant cells have been described in the art:

USP 5,019,504 discloses a method for producing taxol 35 and its derivative utilizing cultured cells of Taxus brevifolia. However, the yield of taxol described therein is 1-3 mg/L, which is insufficient for industrial

application. Besides, the production of taxol by the cell culture is unstable and even when a primary cell of high productivity can be obtained by selection, it is difficult to keep its content by subculturing (see: E. R. M. 5 Wickremesine et al., World Congress on Cell and Tissue Culture (1992)).

USP 5,015,744 teaches a semi-synthetic method from baccatin III, which is a precursor in biosynthesis of taxol. By the use of the plant tissue culture, a raw 10 material for the semi-synthetic process such as baccatin III can be produced, thus the plant tissue culture can also be utilized for taxol production by the above-mentioned semi-synthetic process.

WO 93/17121 offers a method for taxol production by 15 cell culture of Taxus genus plant while changing composition of medium, growth rate, and production rate, etc. In case of Taxus chinensis, 24.1mg/L of taxol can be obtained in 18 days of culture and biomass doubles every 2.5 days.

20 All of these patents describes methods for mass production of taxol by employing batch culture; there is no teaching in said patents on, nor is there anticipated, semi-continuous culture of a taxol-producing cell line.

Under the circumstances, USP 5,407,816 describes that 25 Taxus chinensis cells are inoculated to a nutrient medium to form a suspension which is, in turn, cultivated to form a suspension culture which is, in turn, subcultured in the other nutrient medium to form a producing culture, and which finally gives taxol and taxanes in a yield of 153 30 mg/L. The said method has considerably improved the productivity of taxol, however, it has been proven less satisfactory in the sense that it essentially requires so many nutrient media whose compositions are so complicated, and high productivity can be realized under rather a 35 limited growth condition.

Therefore, there is a continued need to develop a practical and simple method for taxol production, which is

able to meet the requirement of high productivity which is a critical factor to determine whether it can be utilized in industrial applications or not.

5 SUMMARY OF THE INVENTION

In accordance with the present invention, it has been discovered that taxol can be produced with a high degree of efficiency, by the semi-continuous culture of a novel 10 taxol-producing cell line, Taxus chinensis SYG-1.

A primary object of the present invention is, therefore, to provide a method for mass production of taxol by semi-continuous culture.

15 The other object of the invention is to provide a novel taxol-producing cell line isolated from Taxus chinensis.

BRIEF DESCRIPTION OF THE DRAWINGS

20 The above and the other objects and features of the present invention will become apparent from the following descriptions given in conjunction with the accompanying drawings, in which:

25 Figure 1 is a graph showing the effect of AgNO_3 on the taxol productivity.

30 Figure 2 is a graph showing the effect of $\text{NH}_4\text{-citrate}$ on the taxol productivity.

Figure 3 is a graph showing the effect of maltose on the taxol productivity.

35 Figure 4 is a graph showing the productivity of taxol in semi-continuous culture of SYG-1 in accordance with the culture cycle.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors first developed a taxol-producing cell line, based on the callus induced from Taxus chinensis, and compared the cell line with those known in the art. From the comparative studies on the morphological, physiological and growth condition of the taxol-producing cell lines, it has been determined that the newly developed cell line is a novel one which is somewhat different from those of prior art, in light of taxol productivity, mode of taxol secretion, etc. Accordingly, the cell line was named Taxus chinensis SYG-1(hereinafter, referred to as "SYG-1" for convenience) and it was deposited with an International Depositary Authority(IDA), the Korean collection for Type Cultures(KCTC) on March 14, 1996, under an accession number of KCTC 0232BP.

Then, the inventors optimized the growth condition of the cell line of the invention, and determined that: SYG-1 cells grown well on B5 medium, most preferably B5 medium supplemented with 20 μ M NAA(naphthoxyacetic acid), 0.4 μ M BAP(6-benzylaminopurine), 1g/L casein hydrolysate and 30g/L sucrose, under a condition of a temperature of 24°C, at an agitation speed of 150rpm. On the other hand, effects of AgNO₃, NH₄-citrate and maltose on the productivity of taxol were also examined, and the inventors concluded that their addition considerably improved the productivity of taxol.

According to the present invention, taxol can be prepared with a high yield, by employing semi-continuous culture of SYG-1, which comprises:

- (i) inoculating Taxus genus plant cell on a medium containing 1 to 10%(w/v) sugar, and incubating it; and,
- (ii) transferring 1/10 to 1/2 volume of the culture obtained in the step (i) to a fresh medium and repeating the step (i), adding 1 to 10%(w/v) sugar to the remnant culture and incubating to the time of maximum production of

taxol.

At the beginning of culture, AgNO_3 may be added to Taxus genus plant cell culture which is incubated for 10 to 20 days, more preferably 10 to 15 days, at a concentration of 1 to $15\mu\text{M}$, more preferably 5 to $10\mu\text{M}$. Furthermore, NH_4 -citrate and maltose may be added to the culture after 5 to 30 days, more preferably 5 to 10 days of incubation, at a concentration of 1 to 15mM , more preferably 1 to 10mM and 1 to 10% (w/v), more preferably 1 to 5% (w/v), respectively.

After 5 to 30 days of incubation, 1/10 to 1/2 volume of total culture is transferred to other flask containing fresh medium whose contents are the same as the one employed at the beginning of the culture, and started a cycle of culture. Then, remnant culture equivalent to 9/10 to 1/2 volume of total culture, was added 1 to 10% (w/v), more preferably 1 to 5% (w/v) of maltose, and incubated for 30 to 60 days in which taxol production is maximized. At this time, incubation was made at 24°C , agitation speed of 150rpm under a dark condition.

Taxus genus plant employed in the method of the present invention includes Taxus brevifolia, Taxus canadensis, Taxus cuspidata, Taxus baccata, Taxus globosa, Taxus floridana, Taxus wallichiana, Taxus media and Taxus chinensis, however, Taxus chinensis SYG-1 which is newly developed by the inventors, is most preferably employed therein.

According to the method of present invention, the productivity of taxol to the level of 300mg/L, after 42 to 49 days incubation, is observed, when Taxus chinensis SYG-1 is employed in the semi-continuous culture with the addition of AgNO_3 , NH_4 -citrate and maltose to improve the productivity of taxol.

35 Quantitative Analysis of Taxol

Taxol which is produced from the culture of Taxus

chinensis SYG-1 according to the method of the present invention, is quantitatively assayed by employing high performance liquid chromatography under a specific condition described in Table 1 below.

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Table 1: Condition for quantitative assay of taxol

Instrument	HPLC(Waters, U.S.A.)
Column	Capcell Pack C ₁₈ UG 120 (length: 250mm, inner diameter: 4.6mm)
Column temp.	40°C
Mobile phase	CH ₃ CN : water(20 ~ 100% gradient)
Fluid speed	1.0 ml/min
Injection volume	10 μ l
Detector	UV(227nm), ATTE = 3

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The present invention is further illustrated in the following examples, which should not be taken to limit the scope of the invention.

20 Example 1: Induction and characterization of callus from *Taxus chinensis*

The cell line of the present invention was isolated from *Taxus chinensis* by way of callus induction. When 25 plant tissue was incubated on a proper medium containing a hormone, undifferentiated cell of callus was induced. Tissues from bark, needles, stams and roots of *Taxus chinensis* were washed with tap water and sterilized with Ca-hypochloride solution for 20 to 30min. Then, the 30 sterilized tissue was washed with distilled water 2 to 3 times, chopped to have a length of 1cm, and transferred to B5 medium(see: Gamborg et al., Can. J. Biochem., 46:417-421(1968)) supplemented with 20 μ M NAA(naphthoxyacetic acid), 0.4 μ M BAP(6-benzylaminopurine), 1g/L casein

hydrolysate and 30g/L sucrose after solidifying with 0.2% (w/w) gelite, and incubated at a temperature of 24 to 26°C for 2 to 6 weeks under a dark condition to induce the callus of interest.

5 The growth medium for callus was selected by transferring the callus to several media well known in the art, e.g., MS(see: Murashige, T. and F. Skoog, F., Physiol. Plant, 5:473(1962)), SH(see: Schenk and Hildebrandt, Can. J. Bot., 50:199-204(1972)), WPM(see:
10 Lloyd and Mccown, Int. Plant Prop. Soc. Proc., 30:421-427(1981)) and B5 media(see: *supra*), respectively, and observing the growth pattern of the callus by naked eye. From the results above, it was determined that the callus showed good growth on B5 medium containing 20μM NAA, 0.4μM
15 BAP and 2g/L casein hydrolysate.

Then, studies on the morphology, physiology and growth condition were made for the cell line thus induced, and compared with those of known in the art; and, the results were summarized in Tables 2 and 3 below. As can be seen in
20 Tables 2 and 3, it was determined that the call line which has been developed in the invention has a distinction over those of prior art, in light of taxol productivity and mode of taxol secretion, etc. Accordingly, the cell line was named Taxus chinensis SYG-1 and was deposited with an
25 International Depositary Authority(IDA), the Korean Collection for Type Cultures(KCTC) on March 14, 1996, under an accession number of KCTC 0232BP.

Table 2: Morphology and growth condition of *Taxus chinensis*
SYG-1

Taxus chinensis SYG-1		Characteristics
5	Size	50-150 μ m
	Mobility	(-)
	Agglutination	Weak
	Aggregation	(+)
10	Adaptation to shear stress	Strong
	Color of culture	Weak brown
	Culture temperature	24°C

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Example 2: Suspension culture of SYG-1

To select the most preferred medium for the suspension culture of SYG-1, the cell line was transferred to MS, SH, 5 WPM and B5 media, respectively, and the growth pattern was observed analogously in Example 1, which resulted that SYG-1 cell was well grown on B5 medium containing 20 μ M NAA, 0.4 μ M BAP and 2g/L casein hydrolysate, like the callus. In addition, it was also determined that SYG-1 cell was well 10 grown at a temperature of 24°C, at an agitation speed of 150rpm.

A serial cultivation of the callus was made every 4 weeks in a case of solidified medium, while transferring a part of tissue by the aid of forceps: A piece of the callus 15 was maintained on the plate of solid medium. Then, the callus maintained on the solid medium was inoculated into a small volume of B5 medium, and small amounts of medium were supplemented to the culture, as the cells grew to increase total volume of culture. Then, well grown cell 20 lines were diluted in the modified B5 medium contained in 500ml Erlenmeyer flask at a ratio of 1/5(v/v), and inoculated into suspension culture medium every two weeks.

Example 3: Effect of AgNO₃ on the taxol productivity

25 It has been well known that AgNO₃ is an antagonist of the plant hormone, ethylene, which effects on the plant cell growth and secondary metabolite production. Accordingly, the present inventors tested the effect of 30 AgNO₃ on the taxol production in a suspension culture of SYG-1.

To a 250ml of Erlenmeyer flask, was poured 75ml of B5 medium containing 10 μ M AgNO₃, and 25ml of 14 day old cell culture was inoculated into this medium, and incubated in 35 an analogous manner described in Example 2. Then, the productivity of taxol was compared with a control which does not contain AgNO₃ (see: Fig. 1). As can be seen in Fig.

1, it was clearly determined that the amount of taxol produced, 98.95mg/L when AgNO_3 was added to a medium(-O-), was 4.7 times the control(-O-).

5 Example 4: Effect of NH_4 -citrate on the taxol productivity

The productivity of taxol in a suspension culture of SYG-1 was monitored, after the addition of NH_4 -citrate. To a 250ml of Erlenmeyer flask was poured 75ml of B5 medium 10 and 25ml of 14 day old cell culture was inoculated into this medium, and incubated at the same growth condition described in Example 2. Then, 5mM of NH_4 -citrate was added to the culture after 9 days of incubation, and the taxol productivity was compared with a control in which NH_4 -citrate was not added(see: Fig. 2). As can be seen in Fig 15 2, it was determined that the amount of taxol produced was about 103.6mg/L when NH_4 -citrate was added to a medium(-O-), which was 4.9 times the control(-O-).

20 Example 5: Effect of maltose on the taxol productivity

It has been well known that the increase of sugar concentration in a plant cell culture leads to an increase in secondary metabolite production. For example, it has 25 been reported that: addition of 3%(w/v) sucrose and 5%(w/v) mannitol has improved the productivity of anthocyanin in callus culture of Daucus carota(see: Knobloch, K. -H., et al., Zeitschrift fur Naturforschung, 35c:55-556(1981)); and, 88mM sucrose and 165mM mannitol heightened the 30 anthocyanin productivity in a suspension culture of Vitis vinifera(see: Rajendran, L., et al., Biotechnology Letters, 14(8):707-712(1992)). On the other hand, the decrease in the growth and the secondary metabolite production were also reported when the amount of sugar over a certain 35 concentration was added, possibly due to osmotic stress(see: Do, C. B., and Cormier, P., Plant Cell Reports, 9:500-504(1990)).

The effect of maltose addition to SYG-1 was evaluated.

To a 250ml of Erlenmeyer flask was poured 75ml of B5 medium and 25ml of 14 day old cell culture was inoculated into this medium, and incubated under the same growth condition described in Example 2. Then, 2 $\frac{1}{2}$ (w/v) and 3 $\frac{1}{2}$ (w/v) of maltose were added to the culture after 9 days and 21 days of incubation, respectively and the productivity of taxol was compared with a control which does not contain maltose(see: Fig. 3). As can be seen in Fig. 3, it was determined that the amount of taxol was 112.75mg/L when maltose was added to a medium(- \bullet), which was 5.3 times the control(-O-).

Example 6: Semi-continuous culture of SYG-1

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Based on the results obtained in Examples 2 to 5, taxol was prepared by employing semi-continuous culture of SYG-1.

To a 250ml of Erlenmeyer flask was added 80ml of growth medium and 20ml of 14 day old SYG-1 culture, 10 μ M AgNO₃ was added at the beginning of culture, followed by the addition of 5mM NH₄-citrate and 2 $\frac{1}{2}$ (w/v) maltose after 9 days of incubation. After 21 days of incubation, 20ml of culture equivalent to 1/5 volume of total culture was transferred to other flask containing 80ml fresh medium to start another cycle of culture. Then, to 80ml of remnant culture equivalent to 4/5 volume of total culture, was added 3 $\frac{1}{2}$ (w/v) maltose, and incubated for additional 21 days in which taxol production is maximized. At this time, incubation was made at 24°C, and an agitation speed of 150rpm. Culture samples were taken, periodically to check for microbial contamination. The amount of taxol produced in the culture was also determined by employing high performance liquid chromatography(HPLC, Waters, U.S.A.) described as above. As a result, microbial contamination was not observed and the productivity of taxol after 42 days incubation was determined to be 284mg/L(see: Fig. 4).

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As can be seen in Fig. 4, taxol concentration was increased as the cycles of culture were repeated.

As clearly illustrated and demonstrated as above, the 5 present invention provides a method for mass production of taxol by semi-continuous culture of Taxus genus plant with a high yield. According to the present invention, the productivity of taxol to the level of 300mg/L, after 40 to 10 50 days incubation, was observed, when Taxus chinensis SYG-1 was employed in the semi-continuous culture with the addition of AgNO_3 , NH_4 -citrate and maltose to improve the productivity of taxol.

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WHAT IS CLAIMED IS:

1. A method for mass production of taxol by semi-continuous culture which comprises the steps of:

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(i) inoculating Taxus genus plant cell on a medium containing 1 to 10% (w/v) sugar, and incubating it; and,

10 (ii) transferring 1/10 to 1/2 volume of the culture obtained in the step (i) to a fresh medium and repeating the step (i), adding 1 to 10% (w/v) sugar to the remnant culture and incubating to the time of maximum production of taxol.

15 2. The method of claim 1, wherein the Taxus genus plant is selected from the group consisting of Taxus brevifolia, Taxus canadensis, Taxus cuspidata, Taxus baccata, Taxus globosa, Taxus floridana, Taxus wallichiana, Taxus media and Taxus chinensis.

20

3. The method of claim 1, wherein the Taxus genus plant cell is Taxus chinensis SYG-1 (KCTC 0232BP).

25 4. The method of claim 1, which further comprises a step of addition of AgNO_3 to the medium at a concentration of 1 to 15 μM at the beginning of cell culture.

30 5. The method of claim 1, which further comprises a step of addition of NH_4 -citrate or maltose, or both, to the medium at a concentration of 1 to 15 mM and 1 to 10% (w/v), after 5 to 30 days of incubation, respectively.

35 6. The method of claim 1, wherein the time of maximum production of taxol is 40 to 50 days from the beginning of cell culture.

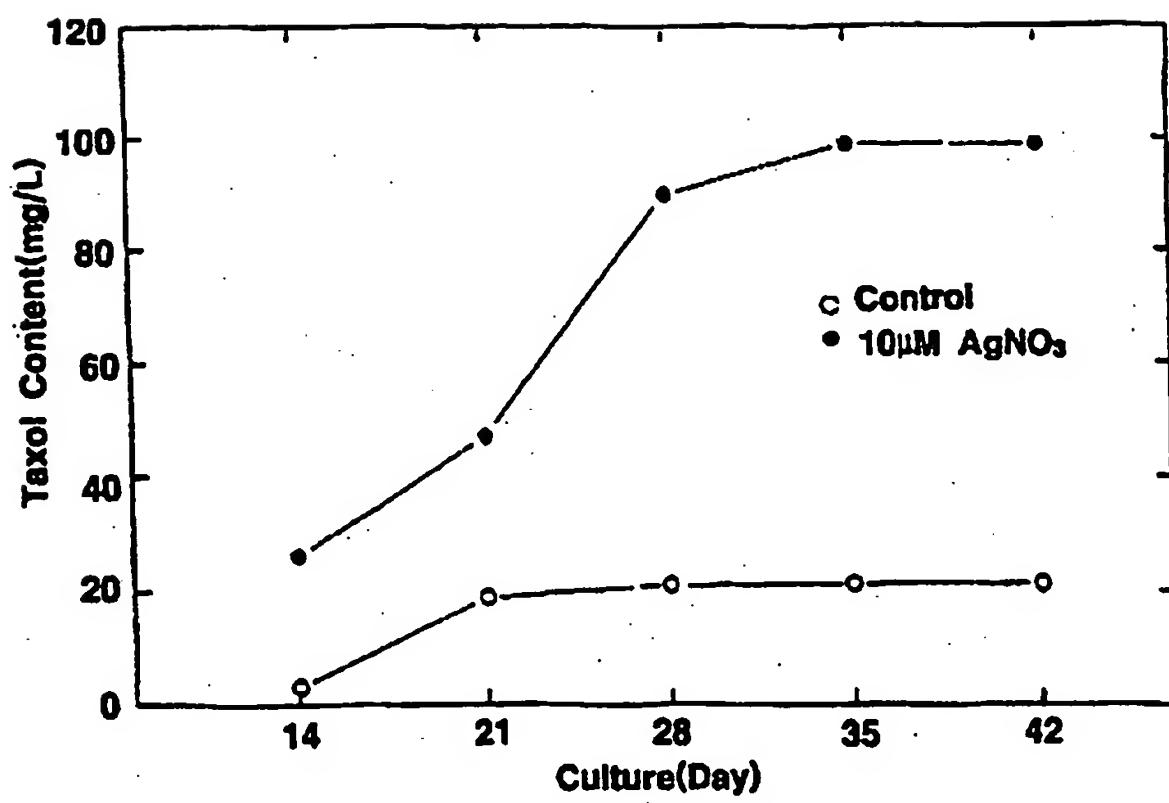


FIGURE 1

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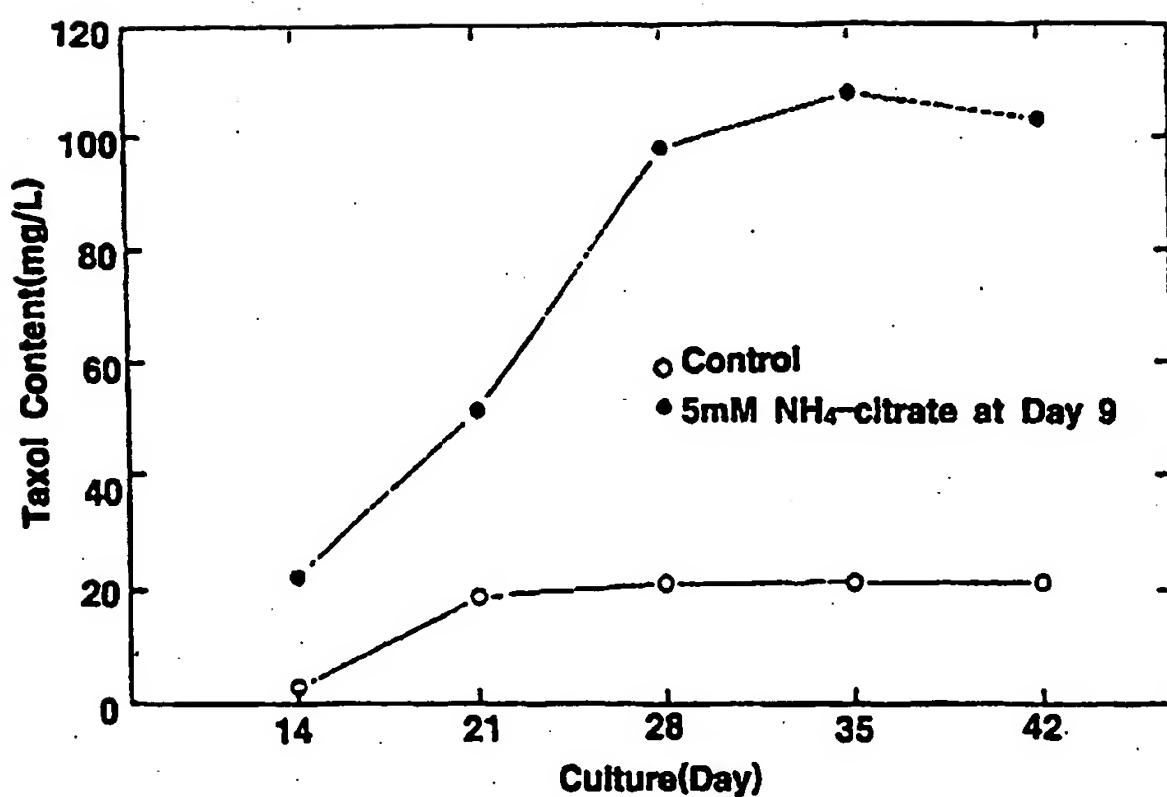


FIGURE 2

3/4

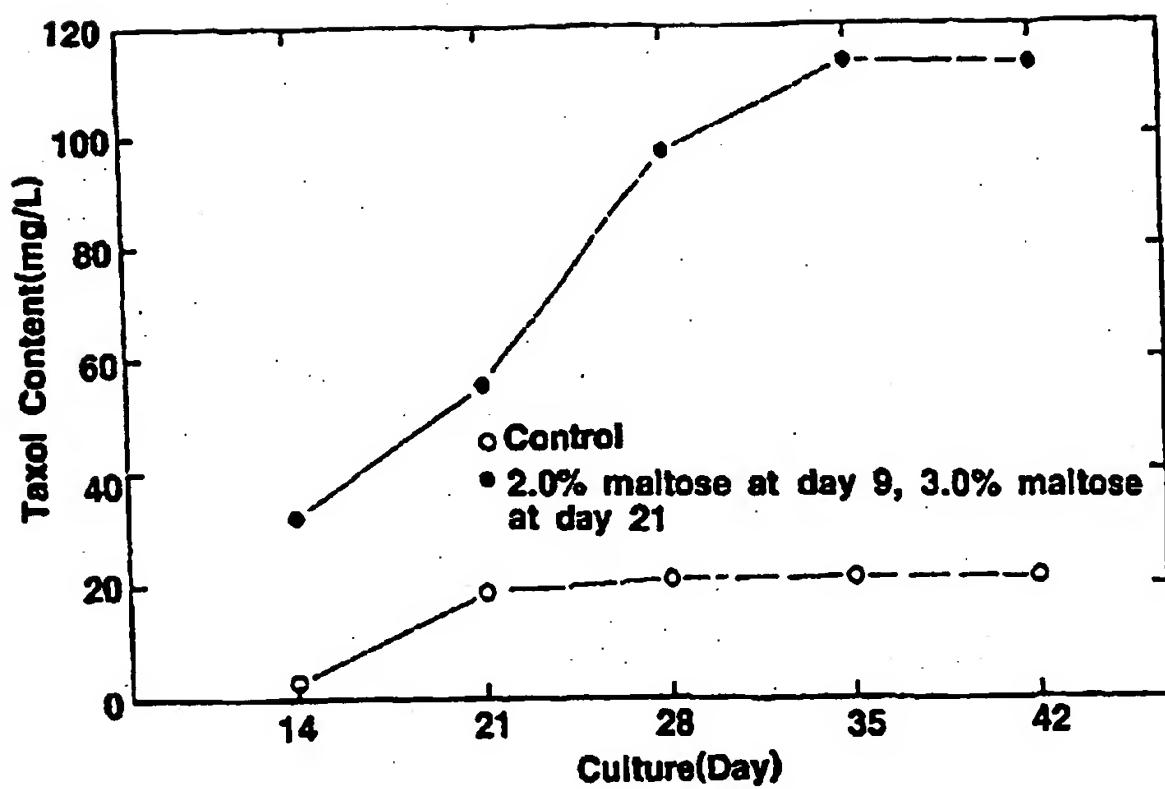


FIGURE 3

4/4

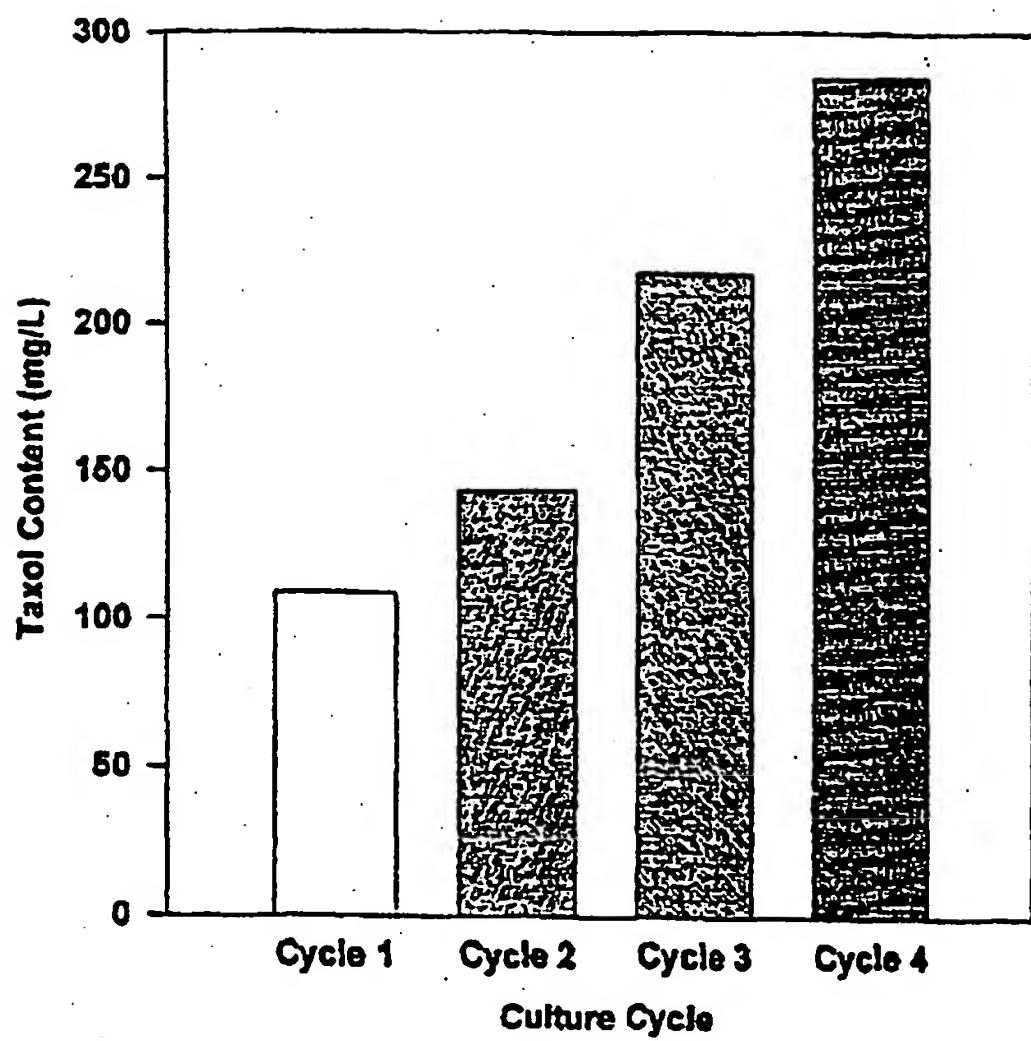


FIGURE 4

KT/KR 96 / 00060

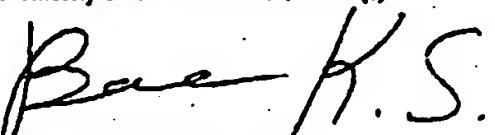
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INTERNATIONAL FORM

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Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: <i>Taxus chinensis STG-1</i>	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY: <i>KCTC 0232BP</i>
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: <input checked="" type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
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IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depository Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on	
V. INTERNATIONAL DEPOSITORY AUTHORITY	
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Address: KCTC, KRIBB #52 Oein-dong, Yusong-ku, Taejon 305-600, Republic of Korea	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 96/00060

A. CLASSIFICATION OF SUBJECT MATTER

IPC⁶ : C 12 P 17/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC⁶ : C 12 P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93/19 585 A1 (UNION CAMP CORPORATION) 14 October 1993 (14.10.93), claims 1,2,12,13; page 23.	1,2
A	WO 93/17 121 A1 (PHYTON CATALYTIC, INC.) 02 September 1993 (02.09.93), claims 1,9,21,22.	1-3
A	US 5 019 504 A (CHRISTEN et al.) 28 May 1991 (28.05.91), claims 1-4,9. -----	1

 Further documents are listed in the continuation of Box C. See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
08 July 1996 (08.07.96)	18 July 1996 (18.07.96)
Name and mailing address of the ISA/AT AUSTRIAN PATENT OFFICE Kohlmarkt 8-10 A-1014 Vienna Facsimile No. 1/53424/535	Authorized officer Wolf Telephone No. 1/53424/133

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/KR 96/00060

In Recherchenbericht angeführtes Patentdokument Patent document cited in search report	Datum der Veröffentlichung Publication date	Mitglied(er) der Patentfamilie Member(s) Member(s) of the family of patents	Datum der Veröffentlichung Publication date
Document de brevet cité dans le rapport de recherche	Date de publication	Members(s) de la famille de brevets	Date de publication
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